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EFFECT OF TYPE OF MATURE EMBRYO EXPLANTS AND ACETOSYRINGONE ON AGROBACTERIUM-MEDIATED TRANSFORMATION OF MOROCCAN DURUM WHEAT

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Abstract

Drought is one of the major constraints in durum wheat production in the Mediterranean Basin. In order to overcome this problem, the genetic transformation of durum wheat is one of the choices for improvement. However, the recalcitrance to *Agrobacterium*-mediated transformation in durum wheat (*Triticum turgidum* L.) is one of the factors limiting a successful genetic transformation. The aim of this study was to investigate the effect of explant type and acetosyringone concentration for the efficient *Agrobacterium*-mediated genetic transformation of three Moroccan durum wheat varieties (Amria, Chaoui, and Marouane). The mature embryos (intact, halved and pieces) were inoculated with *Agrobacterium tumefaciens* strain EHA101 harboring the binary vector pTF101.1 containing drought tolerance gene *HVA1 from barley*, and a selectable marker phosphinothricin (PPT) resistance (*bar*) gene. The explants were inoculated with *A. tumefaciens* (cell density OD_{650} at 0.7) at four different concentrations of acetosyringone (0, 100, 200, and 400 µM). The results showed that embryogenic calli from mature embryos showed higher regeneration and transformation than mature embryo halves and pieces. The integration of the transgene was confirmed by PCR amplification using primers specific to the *bar* gene, *2x35S* promoter, and *HVA1* gene. The transformation efficiency ranging from 0.33% to 2.33% was obtained in Amira variety using embryogenic calli and acetosyringone concentrations of 200 and 400 µM. The integration, as well as inheritance of the transgene, was confirmed by PCR amplification in T_0 and T_1 generations. This is the first report describing a genetic transformation of Moroccan durum wheat varieties via *Agrobacterium tumefaciens.*

Keywords: *Agrobacterium tumefaciens*. Drought Tolerance. Genetic Transformation. *HVA1* Gene. *Triticum turgidum* L.

1. Introduction

The Mediterranean countries' main food crop is durum wheat (*Triticum turgidum* L. *var. durum*). Various biotic and abiotic stresses limit durum wheat production in these countries. As a result, the yield levels are low in many of the countries including Morocco. Durum wheat production in Morocco is reducing year after year, due to low rainfall (drought) and biotic stresses. For instance, Morocco produced 1.3 million tons of durum wheat during the 2018-19 season (Reuters 2018; Usda-Fas 2019) which is 43% less than the previous year. This year again durum wheat production is expected to reduce further by 60% compared to the previous year due to these stresses (World-Grain 2020). As a consequence, Morocco is not self-sufficient

in durum wheat production. Among abiotic stresses, drought and salinity stresses are important in many countries including Morocco. In addition to classical plant breeding, genetic transformation had been shown to be useful in enhancing tolerance to drought in wheat.

Since the first transgenic wheat using the microprojectile bombardment on bread wheat (Vasil et al. 1991; Vasil et al. 1992) and durum wheat (Bommineni et al. 1997) using isolated scutella as explant, *GUS* as a reporter gene, and *bar* as a selectable marker, several improvements have been made in the durum wheat transformation protocol (Iraqi et al. 2005; He et al. 2010; Hakam et al. 2016). However, the first successful *Agrobacterium tumefaciens* mediated transformation for durum wheat using mature and immature embryos was reported by Patnaik et al. (2006) and Wu et al. (2007). Several studies demonstrated that *Agrobacterium*-mediated transformation has been a better alternative to biolistic for low copy insertions of T-DNA with minimal rearrangements and degree of gene-silencing phenomenon (Cheng et al. 2004; Jones et al. 2005; Shrawat and Lörz 2006) and improved stability of expression over generations than the free DNA delivery methods (DAI et al. 2001). The development of a robust *Agrobacterium*-mediated transformation protocol for a recalcitrant species like durum wheat requires the identification and optimization of factors (genotype, type of explant, optimized conditions of inoculation and co-cultivation, selection media, vector, and *Agrobacterium* strain) affecting T-DNA delivery and plant regeneration have been explored in a quest to achieve higher transformation efficiency (Wu et al. 2003).

In our study, barley *HVA1* gene encoding a late embryogenesis abundant (LEA) protein, which confers osmotolerance for seed embryos as an adaptive response during cellular dehydration in seed was used to transform durum wheat. This gene has appeared as an attractive candidate for the engineering of drought and improving growth characteristics including an enhanced biomass yield under water deficit conditions. The aim of this study is to examine the combined effects of several factors influencing *Agrobacterium*-mediated transformation such as genotype, type of explant, and acetosyringone concentration to define the optimum conditions using mature embryos as starter explants for introducing the barley *HVA1* gene into Moroccan durum wheat varieties.

2. Material and Methods

Plant material

Amria, Chaoui, and Marouane, three Moroccan durum wheat varieties, were used for genetic transformation. The seeds of these varieties were procured from the Experimental Research Station of INRA, Marchouch, Morocco. Seeds were disinfected for one minute with 70 % ethanol (v/v), then immersed in 20 % commercial bleach (with 0.1 % Tween 20) for 20 minutes, followed by three rinses with sterile distilled water. Inoculation of mature embryos with *Agrobacterium* cells was performed using three different methods under aseptic conditions:

- Embryogenic calli (EC) were prepared by culturing the intact mature embryos on the induction medium for 15 days in the dark at 25 °C before inoculation with *Agrobacterium*.
- The mature embryo halves (MEH) were prepared by cutting mature embryos into two halves along the embryonic axis and used directly for inoculation with *Agrobacterium*.
- The mature embryo pieces (MEP) were prepared by gently scrapping mature embryos into small pieces with a sharp blade and cultured on the induction medium for 7 days in the dark at 25 °C before inoculation with *Agrobacterium*.

The prepared explants were incubated in MS medium (Murashige and Skoog 1962) supplemented by 100 mg/L Myo-inositol, 150 mg/L asparagine, 20 g/L saccharose, and 2 mg/L picloram.

Agrobacterium mediated gene transfer in mature embryos of wheat

Agrobacterium tumefaciens strain EHA101 harboring pTF101.11 plasmid, containing the *HVA1* gene and *bar* gene which confers resistance to the phosphinothricin (PPT) described by Abdelwahd et al. (2013) were used in this study.

For the preparation of the bacterial suspension culture, 1 mL of *A. tumefaciens* (EHA101) culture was allowed out from a -80 °C glycerol stock culture and precultivated on Yep solid medium containing 50 µg/mL

spectinomycin at 28 °C for 1 to 2 days. Single colonies of the bacteria (EHA101) were grown overnight (48 h) on a shaker at 28 °C and 250 rpm in YEP liquid medium containing appropriate antibiotics. The bacterial cell suspensions were centrifuged at 4,500 x g for 10 minutes. The supernatant was eliminated, and the pellet was resuspended in inoculation medium WWC described by (Wang et al. 2009) with different concentrations of acetosyringone (AS) (0, 100, 200, and 400 µM). The cell concentration was adjusted to an OD_{650} at 0.7. The explant EC, MEH, and MEP were immersed in suspension cells bacteria with AS at different concentrations (0, 100, 200, and 400 µM) for 30 minutes in dark at room temperature. After removing the bacterial suspension, the explants were blotted to dry with sterile filter paper and transferred to fresh co-cultivation medium (i.e., previous induction medium) supplemented by 100 mg/L casein hydrolysate (CH), 2.0 mM MES and 0.75 g/L $MgCl_2$, and maintained in the dark for three days. Subsequently, the embryos were transferred to the same medium plus 300 mg/L of cefotaxime to remove *Agrobacterium*. After 3-4 weeks in the dark, the calli were transferred onto a regeneration medium (Iraqi et al. 2005) and grown for two weeks. Then, the plantlets were transferred to a selective regeneration medium supplemented with 100 mg/L casein hydrolysate, 3 mg/L of PPT, and 300 mg/L of the antibiotic cefotaxime.

DNA analysis

Genomic DNA was extracted from leaves of the putative transformed and untransformed control plants using a CTAB DNA isolation method (Udupa et al. 1998). PCR amplification in a 20µL solution containing 50 ng DNA template, 1x Taq DNA polymerase buffer, 200 µM of each dNTP, 0.5 pmol of the respective primers, and 0.6 unit of Taq DNA polymerase revealed the presence of the *bar* gene, *2x35S* promoter, and *HVA1* gene sequences in all putative transgenic plants (Promega). 5' GTCTGCACCATCGTCAACC 3' (forward) and 5' GAAGTCCAGCTGCCAGAAAC 3' (reverse) primer pairs were used to detect the *bar* gene (402 bp). 5' GCACAATCCCACTATCGTTCGC 3' (forward) and 5' TCCGTCCACTCCTGCGGTTC 3' (reverse) primer pairs were used to detect the *2x35S* (390 bp) gene. Both primers (*bar* and *2x35S*) were amplified in a thermocycler (Master Cycler, Eppendorf, Germany) with an initial denaturation at 94 °C for 4 minutes, followed by 30 cycles of 1 minute at 94 °C, 1 minute at 58 °C, 2 minutes at 72 °C, and a final 10 minute extension at 72 °C. The primer pairs used to detect the *HVA1* gene (290 bp) were 5' AGCTAGATCGTGTGAGACGAAG 3' (forward) and 5' CCTGCGCCGTCTCGTACGTCTT 3' (reverse), *HVA1* primers were amplified using an initial 3 minute denaturation at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55.5 °C, 75 seconds at 72 °C, and a final 10 minute extension at 72 °C. PCR products were separated on 1% (w/v) agarose gel and 8% (w/v) polyacrylamide gel and stained with ethidium bromide. The size of the amplified transgene product was compared to that of the positive control.

Statistical analysis

A completely randomized design (RCBD) was used with 3 genotypes (Amria, Chaoui, and Marouane), 4 concentrations of AS (0, 100, 200, and 400 µM), and three types of mature embryo explants (ME, MEH, and MEP). Each test was performed three times. For each treatment, we used 100 explants per replication. The percentage of callus induction, plantlets regeneration, and resistant plantlets to selective agent phosphinothricin (PPT) were counted. Analysis of Variance (ANOVA) was performed using the General Linear Model (GLM) procedure in SAS (SAS Institute 1985). Fisher's Least Significant Difference (LSD) test was used to explore further and compare the mean of treatments (SAS Institute 1985).

3. Results

The experiments were conducted to evaluate the effect of the factors influencing the efficiency of T-DNA delivery in durum wheat known by recalcitrance to *Agrobacterium*-mediated transformation. These factors include genotype, four different concentrations of AS (0, 100, 200, and 400 µM), and different explants (embryogenic calli, mature embryo halves, and mature embryo pieces).

Effect of genotype on the regeneration and transformation efficiency

The mature embryos have been used for evaluating the callus induction and regeneration after infection by *Agrobacterium* and transformation response in three durum wheat Amria, Chaoui, and Marouane. 3600 explants were infected by *Agrobacterium* cells for each variety and the analysis of variance clearly showed a significant difference between varieties (Table 1) on callus induction and regeneration after infection with bacteria cells. The callus induction (79.13%) and plantlets regeneration (9.37%) (Table 1) for the Amria variety were higher compared to Chaoui and Marouane. Although the results of plantlets resistant to PPT showed no significant difference between varieties, the transformation efficiency for variety Chaoui (0.06%) was lower than Amria (0.25%) and Marouane (0.11%). In fact, Amria and Marouane were the most sensitive varieties to *Agrobacterium* infection than Chaoui (Table 1).

Table 1. Effect of genotype on callus induction, regeneration and transformation efficiency in durum wheat.

Varieties	Callus induction (%)	Plantlets regeneration (%)	Plantlets resistant to PPT (%)	Transformation efficiency (%)
Amria	79.13^{a*}	9.37^{a}	1.12^{a}	0.25^{a}
Chaoui	75.42^{b}	7.24^{b}	1.05^{a}	0.06^{b}
Marouane	73.59^{b}	9.35^{ab}	1.38^{a}	0.11^{a}
LSD	2.193	2.1287	1.0631	0.165

*The values followed by the same alphabet are not significantly different at alpha =0.05 according to the Least Significant Difference (LSD) test.

Effect of acetosyringone concentration on callus induction, regeneration plant, and transformation efficiency

The three explants (embryogenic callus, mature embryo halves, and mature embryos pieces) were inoculated for 30 minutes in the presence of *Agrobacterium* cells with different concentrations of AS 0, 100, 200, and 400 µM. The presence of virulence inducers such as AS in the inoculation media is necessary to improve the efficiency of T-DNA transfer in durum wheat because of its resistance to *A. tumefaciens* infection. The results revealed that different concentrations of AS had no significant effect on callus induction and plantlets regeneration. However, the inoculation media with 100 and 400 µM gave the highest percentage of plantlets resistant to PPT (1.68%) and (1.56%) respectively compared to 0 and 200 µM (Table 2). The results of transformation efficiency showed a significant difference between the four AS concentrations, inoculation medium containing 100 and 400 µM gives higher transformation efficiency (0.26%) compared to 200 µM (0.04%) (Table 2).

Table 2. Effect of acetosyringone concentration on callus induction, regeneration and transformation efficiency in durum wheat.

AS (µM)	Callus Induction (%)	Plantlets Regeneration (%)	Plantlets Resistant to PPT (%)	Transformation efficiency (%)
000	77.52^{b*}	7.74^{b}	0.94^{ab}	0^{b}
100	76.17^{b}	8.56^{b}	1.68^{a}	0.26^{a}
200	75.12^{b}	9.67^{b}	0.54^{ab}	0.04^{b}
400	75.38^{b}	8.65^{b}	1.56^{a}	0.26^{a}
Control	81.30^{a}	20.83^{a}	0^{b}	0^{b}
LSD	2.9401	2.8343	1.405	0.1912

*The values followed by the same alphabet are not significantly different at alpha =0.05 according to the Least Significant Difference (LSD) test; AS: Acetosyringone.

Effect of explant on callus induction, regeneration, and transformation efficiency

Among the three explants used, embryogenic calli showed the highest values on callus induction (100%) in comparison to MEH and MEP explant. However, the highest regeneration rate was obtained using mature embryo halves as explant (10.6%) (Table 3). The selection results indicated that the plantlets resistant to selective agent PPT differed significantly among explants ($p<0.001$). In fact, embryogenic calli

recorded the highest value (2.41%) of resistant plantlets followed by mature embryos pieces with 0.79% and mature embryo halves with 0.36%. While transgenic plants were obtained only when using embryogenic calli as started explant with a transformation efficiency of 0.42%. The results suggested that embryogenic callus was the best explant to be used as a receptive tissue from the mature embryos for *A. tumefaciens* infection.

Table 3. Effect of explant on callus induction, regeneration and transformation efficiency in durum wheat.

Explant	Callus induction (%)	Plantlets regeneration (%)	Plantlets resistant to PPT (%)	Transformation efficiency
EC	100[a*]	7.688[b]	2.406[a]	0.415[a]
HME	63.257[b]	10.6[a]	0.354[b]	0[b]
MEP	64.882[b]	7.676[b]	0.785[b]	0[b]
LSD	2.1931	2.1287	1.0631	0.3918

*The values followed by the same alphabet are not significantly different at alpha =0.05 according to the Least Significant Difference (LSD) test.

Durum wheat is known for its low regeneration capacity. The transformation by *Agrobacterium* reduced further callus induction and plantlets regeneration for all varieties compared to the control. The results revealed that the Amria genotype was a more sensitive genotype to *Agrobacterium* infection than the others when the embryogenic callus was used as starting materials for the transformation. The highest transformation efficiency was observed in the variety Amria (2.33%) at 400 µM of AS followed by 100 µM (0.8%) and 200 µM (0.33%) concentration of AS. Moreover, the transformation efficiency was also highest in variety Chaoui (0.33%) and Marouane (1.33%) using 100 µM as a concentration of AS (Table 4). The callus induction and regeneration were significantly influenced by the type of explant (Table 5).

Table 4. Effect of explant embryogenic callus (EC) and their interaction with genotype and acetosyringone (AS) concentration on plantlets regeneration, plantlets resistant to PPT, and transformation efficiency in durum wheat.

AS (µM)	Number of EC infected	Plantlets regeneration (%)	Plantlets resistant to PPT (%)	Transformation efficiency (%)
		Amria		
0	300	3.56[de*]	0.79[c]	0[c]
100	300	8.54[cde]	1.97[bc]	0.3[bc]
200	300	5.27[cde]	3[bc]	0.33[bc]
400	300	13.92[bc]	7.52[ab]	2.33[a]
Control	--	23.75[a]	0[c]	0[c]
		Chaoui		
0	300	5.01[de]	1.97[bc]	0[c]
100	300	3.42[de]	1.18[c]	0.66[bc]
200	300	5.52[cde]	2.77[bc]	0[c]
400	300	1.52[e]	0[c]	0[c]
Control	--	22.25[ab]	0[c]	0[c]
		Marouane		
0	300	11.67[cd]	1.18[c]	0[c]
100	300	23.57[a]	11.5[a]	1.33[ab]
200	300	4.73[de]	0[c]	0[c]
400	300	5.52[cde]	0[c]	0[c]
Control	--	26.25[a]	0[c]	0[c]
LSD		8.8924	6.2446	0.4147

*The values followed by the same alphabet are not significantly different at alpha =0.05 according to the Least Significant Difference (LSD) test; AS: Acetosyringone.

Table 5. Analysis of variance for effects of variety, explant, acetosyringone concentration (ConAS) and their interaction on callus induction, plantlets regeneration (%), plantlets resistant to PPT, and transformation efficiency (%) in durum wheat.

	Callus induction (%)	Plantlets regeneration (%)	Plantlets resistant to PPT (%)	Transformation efficiency (%)
Explant	540.73***	5.45**	8.36***	11.93***
Variety	12.29***	3.43*	0.05	2.01
Explant*variety	8.11***	3.94**	0.71	2.01
Concentration of AS (ConAS)	1.47	0.97	1.07*	0.04
Explant*ConAS	1.73**	4.42***	2.14*	2.45*
Variety*ConAS	2.76*	1.01	2.44*	1.89
Explant*variety*ConAS	2.32**	2.15*	3.22***	1.89*

*Significant at p<0.05; **Significant at p<0.01; ***Significant at p<0.001; ConAS: Concentration of Acetosyringone.

Molecular analysis of transgenic plants and inheritance of transgenes

PCR analysis was performed to confirm the presence of the *bar* gene, *2x35S* promoter, and *HVA1* gene in genomic DNA isolated from the putative transgenic plants T0. 98 plants were obtained from all treatments, 9 plants obtained from mature embryos pieces, and 14 plants from mature embryo halves. No plant among those has integrated the *HAV1* gene into their genome. Among 75 plants issue from embryonic callus, 14 showed amplification with *bar* gene, *2x35S* promoter, and *HVA1* gene (Figure 1). Using embryogenic call as explant the data indicated that transgenic plants expressing *HVA1* gene were successfully produced via *Agrobacterium* mediated transformation of Moroccan durum wheat with a transformation efficiency of 18.6% calculated by (Number of *HVA1* gene-positive plants/Total PPT resistant plants) x 100 and a transformation efficiency of 0.42% calculated as (Number of *HVA1* gene-positive plants/Total number of callus infected) x 100.

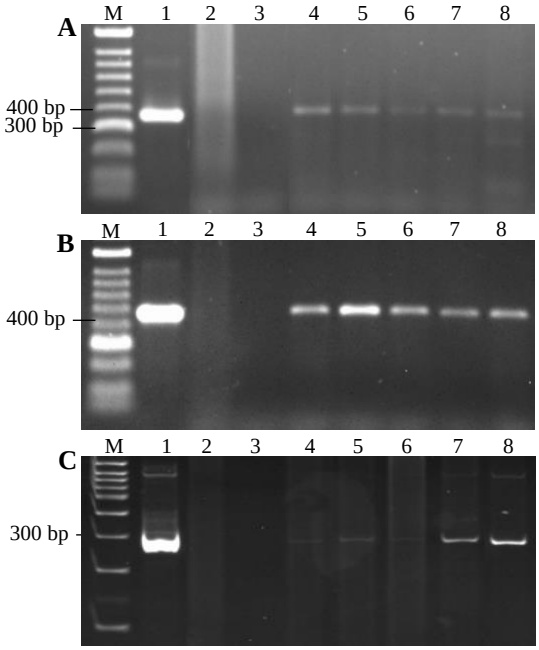

Figure. 1 PCR analysis of genomic DNA samples of T_0 transformants of durum wheat: A – using primers specific to *2x35S* promoter (390 bp)*;* B – using primers specific to *bar* gene (402 bp); and C – using primers specific to *HVA1* gene (290 bp). The lanes M, molecular weight marker (50 bp); 1, plasmid (pTF101.1); 2, non-transformed control; 3, Water control; and lanes 4–8, PCR products of transgenic plants.

Among the 14 transgenic T_0 plants, 9 plants were found to be fertile, PCR amplification was further undertaken to screen for the presence of *bar* gene, *2x35S* promoter, and *HVA1* gene in the T_1 progeny. In 5 of 9 T_1 plants, *bar gene*, *2x35S* promoter, and *HVA1* gene were integrated (Figure 2).

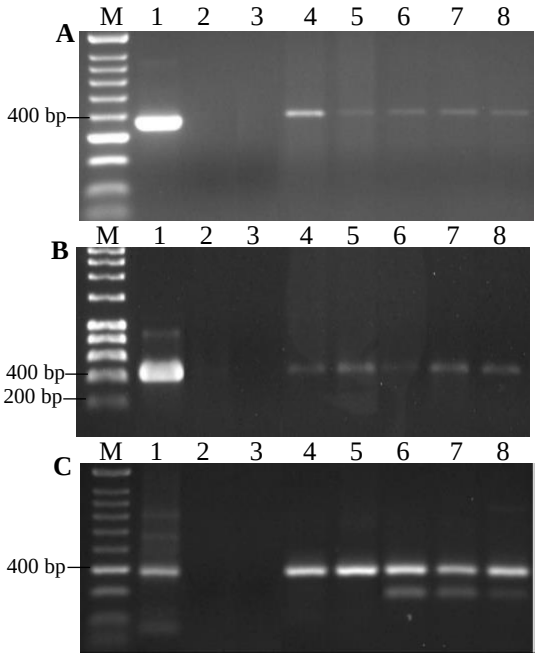

Figure. 2 PCR analysis of genomic DNA samples of T_1 transformants of durum wheat: A – using primers specific to *2x35S* promoter (390 bp)*;* B – using primers specific to *bar* gene (402 bp); and C – using primers specific to *HVA1* gene (290 bp). The lanes M, molecular weight marker (50 bp); 1, plasmid (pTF101.1); 2, non-transformed control; 3, Water control; and lanes 4–8, PCR products of transgenic plants.

4. Discussion

Durum wheat is considered as one of the most recalcitrant species for *in vitro* regeneration and *Agrobacterium*-mediated transformation, mainly due to the low efficiency of plant regeneration under tissue culture conditions. Since the first transgenic bread wheat plant by *Agrobacterium*-mediated transformation using immature embryos (Cheng et al. 1997), more research focused on transgenic wheat via *Agrobacterium* had been given. However, *Agrobacterium*-mediated transformation of wheat is known to be affected by different factors including genotype, explant, AS concentration. The present investigation was initiated to develop an efficient gene delivery protocol for durum wheat using three explants (embryogenic calli, mature embryo halves, and mature embryo pieces) derived from mature embryos of Moroccan durum wheat varieties of Amria, Chaoui, and Marouane; and four different concentrations of AS (0, 100, 200 and 400 µM).

Our results showed that the plantlets' regeneration and transformation efficiency was significantly influenced by explant. The transgenic plants were obtained only when using embryogenic calli as explant and no transgenic plant was recorded using mature embryo halves and mature embryos pieces. This is not in line with the results of Wang et al. (2009) where mature embryo halves explant was generally more suitable for transformation, with a frequency of 0.12% to 1.79%, for bread wheat varieties (Yumai 66, Lunxuan 208 and Bobwhite). Other studies had also shown that the frequencies of resistant plantlets were higher using MEH (0.53 to 11.62%) compared to EC (0 to 6.93%) (Aadel et al. 2018). Patnaik et al. (2006) successfully generated transgenic durum wheat through *Agrobacterium* mediated transformation with the frequency of 1.28% and 1.54% using the constructs LBA4404 (pBI101/Act1) and LBA4404 (pCAMBIA/*pin2*) respectively with mature embryo-derived callus. In our study, the transformation frequency ranged from 0.33% to 2.33% using embryogenic calli in the three varieties of durum wheat (Amria, Chaoui, and Marouane). This percentage is higher compared to the result obtained by Wang et al. (2009) and Patnaik et al. (2006). Furthermore, the average rate of transformation was higher than those reported for Moroccan durum wheat using mature embryos and biolistic transformation (0.66%; Hakam et al. 2016). Other results revealed a higher transformation efficiency (average 3.1%) of durum wheat cv. Ofanto with a superbinary pGreen/pSoup system (Wu et al. 2007). He et al. (2010) had further improved the transformation efficiency to 6.3% in durum wheat cv. Stewart. The lower value of transformation efficiency obtained in our experiments compared to He et al. (2010) and Wu et al. (2007) could be due to several factors such as the use of a different genotype, explant or transformation conditions.

Our results confirm that AS is necessary for the *Agrobacterium* mediated transformation in durum wheat, which is in line with several other reports in bread wheat (Cheng et al. 1997; Wu et al. 2003; He et al. 2010). In our study, the highest transformation efficiency was observed in the variety Amria (2.33%) at concentration 400 µM of AS. Also, in the case of variety Amira, increase in the concentration of AS, increased transformation efficiency, which is in line with the observation of He et al. (2010) where they suggested that an increase of AS concentration from 200 µM to 400 µM improve final transformation. Another study has also shown that the use of a co-culture medium supplemented with AS at a concentration of 400 µM was the most favorable for T-DNA delivery (Manfroi et al.2015). Aadel et al. (2018) had reported that 200 µM of AS was the optimal concentration for the transformation of bread wheat. In contrary with Rashid et al. (2011), they reported that only 50 µM of AS gives maximum transformation efficiency in wheat.

5. Conclusions

In conclusion, we have successfully introduced the barley *HVA1* gene into Moroccan durum wheat varieties via *Agrobacterium*-mediated transformation using embryogenic calli originating from mature embryos as an explant and using Acetosyringone to improve transformation efficiency. The transformation protocol was developed for the first time in Moroccan durum wheat varieties.

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